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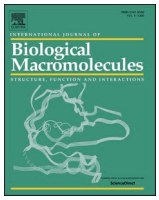

Review

Considerations about the inhibition of monophenolase and diphenolase activities of tyrosinase. Characterization of the inhibitor concentration which generates 50 % of inhibition, type and inhibition constants. A review

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ABSTRACT

Tyrosinase is a copper oxidase enzyme which catalyzes the first two steps in the melanogenesis pathway, L-tyrosine to L-dopa conversion and, then, to *o*-dopaquinone and dopachrome. Hypopigmentation and, above all, hyperpigmentation issues can be originated depending on their activity. This enzyme also promotes the browning of fruits and vegetables. Therefore, control of their activity by regulators is research topic of great relevance. In this work, we consider the use of inhibitors of monophenolase and diphenolase activities of the enzyme in order to accomplish such control. An experimental design and data analysis which allow the accurate calculation of the degree of inhibition of monophenolase activity (i_M) and diphenolase activity (i_D) are proposed. The IC_{50} values (amount of inhibitor that causes 50 % inhibition at a fixed substrate concentration) can be calculated for the two activities and from the values of IC_{50}^{M} (monophenolase) and IC_{50}^{D}(diphenolase). Additionally, the strength and type of inhibition can be deduced from these values. The data analysis from these IC_{50}^{D} values allows to obtain the values of $K_{I_1}^{app}$ or $K_{I_2}^{app}$, or $K_{I_1}^{app}$ and $K_{I_3}^{app}$ from the values of IC_{50}^{M}. In all cases, the values of the different K_I^{app} must satisfy their relationship with IC_{50}^{M} and IC_{50}^{D}.

1. Introduction

Tyrosinase (EC1.14.18.1) is a cuproprotein widely distributed in nature from bacteria, fungi, plants and animals [1]. The enzyme catalyzes two reactions: (i) the hydroxylation of monophenols to *o*-diphenols; and (ii) the subsequent oxidation of *o*-diphenols to *o*-quinones with the participation of molecular oxygen [2]. The correct functioning of tyrosinase turns out to be highly important in order to avoid hyperpigmentation or hypopigmentation processes [3].

Thus, tyrosinase shows two activities in the melanin biosynthesis pathway: (i) the hydroxylation of L-tyrosine to L-dopa; and (ii) the oxidation of L-dopa to L-*o*-dopaquinone. Since *o*-dopaquinone is unstable, it evolves chemically, generating dopachrome and regenerating L-dopa in the medium. These chemical reactions are very relevant since the enzyme is found in three forms in the catalytic cycle: (i) meta-tyrosinase ($Cu^{+2}Cu^{+2}$); (ii) deoxy-tyrosinase ($Cu^{+1}Cu^{+1}$); and (iii) oxy-tyrosinase ($Cu^{+2}Cu^{+2}O_2^{-2}$). The catalytic cycle closes when the enzyme acts on monophenols. The presence of a reducing agent, such as L-dopa, which reduces meta-tyrosinase to deoxytyrosinase is necessary. It should be noted that the last-mentioned molecule has a high affinity for oxygen and generates oxy-tyrosinase in the medium [4].

The control of tyrosinase activity is important from a medical point of view, in cosmetics and also in the browning of fruits and vegetables [5]. The most direct control is done by tyrosinase activity and the use of

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inhibitors. Therefore, the kinetic characterization of these inhibitors turns out to be relevant [6–12].

The mechanism of action of tyrosinase on monophenols (Scheme 1) and *o*-diphenols (Scheme 1, with [M] = 0) is complex [13]. In both cases, the enzymatic stages are coupled with a series of purely chemical reactions, which are of crucial importance in the kinetic mechanism of action of the enzyme on monophenols, since the sequence of reactions involves the accumulation of *o*-diphenol in the medium. As shown in Scheme 1, when monophenolase activity is studied, the diphenolase activity is always coupled. The system reaches the stationary state when the quantitative relationship $[D]_{ss}/[M]_{ss} = R$ [14] is satisfied, where $[D]_{ss}$ y $[M]_{ss}$ are the concentrations of *o*-diphenol and monophenol at steady state.

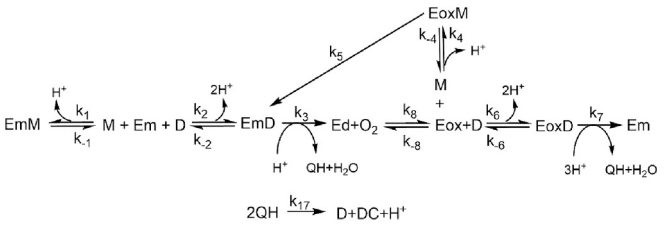
The above-described information implies that the monophenolase activity on its arrival at the steady state is preceded by a delay period (τ), thus making the kinetic study of inhibitors difficult, leading to erroneous data in the bibliography, since it is not evident when the system reaches steady state and there may be errors when determining the rate [15–17]. In previous works, we proposed a method to avoid these interferences. This method involves the addition at $t = 0$ of an amount of *o*-diphenol, which turns out to be necessary in order to instantly reaching the steady state, thus fulfilling the value of the parameter R [14,18].

An additional consideration should be contemplated when measuring the tyrosinase activities: the *o*-quinone reaction product is very reactive and can react with the molecules that are tested as inhibitors [19,20]. This aspect must be controlled in the kinetic characterization of the inhibitors.

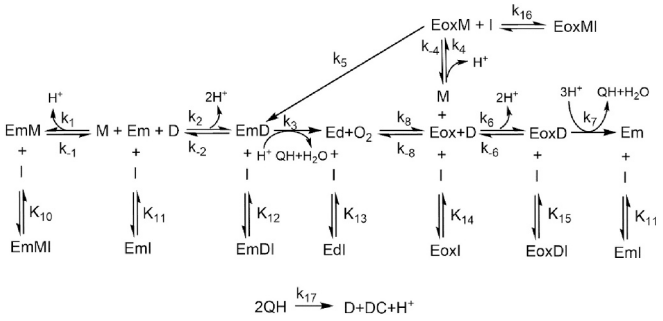
Two parameters are used to kinetically characterized an inhibitor: the strength and the type of inhibition. The strength is indicated by the K_I parameter. The type of inhibition can be divided into competitive, non-competitive, uncompetitive and mixed type M_1 or type M_2 [21,22].

Our group reported the characterization of the action of inhibitors on the diphenolase activity of the enzyme in a previous work (Scheme 2 with [M] = 0) [23]. An experimental design to carry out the study of the strength and type of inhibition on the diphenolase activity of tyrosinase was established. Due to the complex mechanism of the enzyme (see Scheme 1), the absolute inhibition constants cannot be determined using the kinetic study, thus establishing the values of the apparent inhibition constants (K_I^{app}). These values depend on the nature of the employed substrate. An experimental design for the calculation of the IC_{50}^{D} parameter was established. This parameter was calculated from the degrees of inhibition i_D, which is a value related to the apparent inhibition constants that are determined using the analytical expressions described in the Supplementary Material. The above-mentioned work showed that the type and strength of an inhibitor could be deduced with few experiments by determining the IC_{50}^{D} value [23–26].

This review aims to further extend the previous analysis to the monophenolase activity of tyrosinase (Scheme 1). The inhibition is shown in Scheme 2, establishing a relationship between the two activities. An experimental design and data analysis are proposed based on the determination of the IC_{50} values for the monophenolase activity



Scheme 1. Diphenolase activity of tyrosinase (if M = 0). Monophenolase activity of tyrosinase (if M ≠ 0). Where M is monophenol (L-tyrosine), D is *o*-diphenol (L-dopa), QH is L-*o*-dopaquinone protonated, Q is L-*o*-dopaquinone deprotonated and DC is L-dopachrome.



Scheme 2. Generalized inhibition of monophenolase activity of tyrosinase and diphenolase activity of tyrosinase (if M = 0). Where M is monophenol (L-tyrosine), D is *o*-diphenol (L-dopa), QH is L-*o*-dopaquinone protonated, Q is L-*o*-dopaquinone deprotonated and DC is L-dopachrome.

(IC_{50}^{M}) and for the diphenolase activity of the enzyme (IC_{50}^{D}). The comparison of these parameters and their analysis leads to discern the type of inhibition (competitive, non-competitive, uncompetitive or mixed) and to calculate its strength ($K_{I_1}^{app}$, $K_{I_2}^{app}$ and $K_{I_3}^{app}$). In addition, a series of works from the bibliography are discussed in relation to the inhibition of the two enzyme activities and the determination of the IC_{50}^{M} and IC_{50}^{D} parameters and their relationship with K_I^{app}. Since this review is focused on kinetic factors in order to establish a methodology to carry out the abovementioned analysis and, therefore, to obtain reliable information, the particular mechanism of action of each of the inhibitors described will not be considered herein.

2. Discussion

The enzyme tyrosinase shows two activities: monophenolase and diphenolase.

The diphenolase activity is the action of the enzyme on *o*-diphenols (L-dopa) using oxygen and generating *o*-quinones. This reaction reaches steady state in a very short time (when t tends to 0). The rate measurements are made at steady state and, in principle, these measurements should be correct and therefore the kinetic studies on the type and strength of inhibitors on this activity as well.

Monophenolase activity studies the action of tyrosinase on monophenols (L-tyrosine), using oxygen and generating *o*-quinone. The kinetic mechanism of monophenolase activity is more complex than that of diphenolase activity. After accumulating a small amount of *o*-diphenol in the medium, the steady state is reached by the enzyme's action [15]. This means that, in order to reach the steady state, a lag period is required. When the performance of the enzyme is studied in the presence of inhibitors, this lag period becomes longer, and it is not known exactly when the system has reached steady state. This problem is overcome by adding a small amount of *o*-diphenol (*D*) before starting the enzymatic reaction with L-tyrosine (*M*). Thus, the ratio *[D]/[M]* must be constant in kinetic experiments [13].

This review postulates an experimental design to address the abovementioned issue, which is based on experimental evidence from previous works. Furthermore, this review proposes an analysis of kinetic data reported in the literature.

2.1. Diphenolase activity

The mechanism of action of tyrosinase on *o*-diphenols is represented in Scheme 1, with [M] = 0. The enzyme in the catalytic cycle goes through three forms: Em (meta-tyrosinase), Ed (desoxy-tyrosinase) and Eox (oxy-tyrosinase). When acting on L-dopa, the reaction product is *o*-dopaquinone, which chemically evolves towards L-dopachrome and L-dopa (see Scheme 1, with [M] = 0) [13].

2.2. Monophenolase activity

The action mechanism of tyrosinase on monophenols (L-tyrosine) is indicated in Scheme 1 [13]. The presence of the three forms (Em, Ed and Eox) stands out in the action of the enzyme. It should be noted the presence of the EmM complex which is inactive. The sequence of chemical reactions involved in the evolution of *o*-dopaquinone must be highlighted. Through these reactions, L-dopa is accumulated in the medium, facilitating catalysis.

The action of an inhibitor on monophenolase activity is shown in Scheme 2 and on the diphenolase activity (Scheme 2, with [M] = 0).

The difference between monophenolase activity (Scheme 1) and diphenolase activity (Scheme 1 with [M] = 0) is the presence of two enzymatic species that are originated by the action of the enzyme on monophenols (EmM and EoxM). Taking into account that the affinity of the enzyme (Ed) for oxygen is very high [4,27], tyrosinase is saturated by oxygen and therefore, the intermediates originated by Ed are practically non-existent. The inhibition of diphenolase activity is determined by the inhibition constants $K_{I_1}^{app}$ and $K_{I_2}^{app}$ and, in the case of monophenolase activity, the inhibition is characterized by $K_{I_1}^{app}$ and $K_{I_3}^{app}$. Therefore, the following types of inhibition can occur:

Competitive inhibition: in this case $K_{I_2}^{app} \to \infty$ y $K_{I_3}^{app} \to \infty$,then the inhibition is characterized by $K_{I_1}^{app}$ in both activities, this will lead to $IC_{50}^{M} = IC_{50}^{D}$.

Non-competitive inhibition: in this case $K_{I_1}^{app} = K_{I_2}^{app} = K_{I_3}^{app} = K_I^{app}$, then the inhibition is characterized by K_I^{app} that is common to both activities, this implies that $IC_{50}^{M} = IC_{50}^{D}$.

Uncompetitive inhibition: in this case $K_{I_1}^{app} \to \infty$.Two situations can occur that $K_{I_3}^{app} = K_{I_2}^{app}$ or that are different. In the first case, the kinetic characterization is given by $K_{I_2}^{app}$, this implies that $IC_{50}^{M} = IC_{50}^{D}$.. In the second case, the kinetic characterization is given by $K_{I_2}^{app}$ and $K_{I_3}^{app}$ and it will happen that $IC_{50}^{M} \neq IC_{50}^{D}$.

Mixed inhibition: in this case $K_{I_3}^{app} \neq K_{I_2}^{app}$. Diphenolase activity is characterized with respect to these inhibitors by $K_{I_1}^{app}$ y $K_{I_2}^{app}$. In the case of monophenolase activity, inhibition is characterized by $K_{I_1}^{app}$ y $K_{I_3}^{app}$. This carries with it $IC_{50}^{M} \neq IC_{50}^{D}$.

Herein, an experimental design based on the relationships between IC_{50}^{M} and IC_{50}^{D} and a data analysis which allows the discrimination between the different action mechanisms of the inhibitors are established, calculating the value of their inhibition strength.

2.3. Proposal of an experimental design to determine the type and strength of tyrosinase inhibitors from the values of i_D and i_M and the values of IC_{50}^{D} and IC_{50}^{M}

The experimental design consists of a series of stages that are established from the determination of the degrees of inhibition i_D and i_M. Firstly, the IC_{50}^{D} and IC_{50}^{M} values are calculated. The comparison between these values allows to easily calculate the type and strength of the action of an inhibitor on the diphenolase and monophenolase activities. The procedure consists of a series of stages.

Step 1. The values V_0 and $V_{0,\,i}$ are calculated from the progress curves of L-dopachrome accumulation in the absence and in the presence of inhibitor. The values of i_D and i_M are calculated by applying Eqs. (S14) and (S21). The ratio $[D]_0$ to K_M^D should be equal to the ratio of $[M]_0$ to K_M^M in order to have values which can be compared. In the case of monophenolase activity, *o*-diphenol must be added to eliminate the lag period of this activity.

Step 2. Representation of the degrees of inhibition i_D vs $[I]_0$ and i_M vs $[I]_0$.

Step 3. Data analysis of i_D vs $[I]_0$ by non-linear regression according to Eq. (S15) and i_M vs $[I]_0$ according to Eq. (S22), which provides the values of IC_{50}^{D} and IC_{50}^{M} for each inhibitor: competitive $IC_{50}^{C,D}$ and $IC_{50}^{C,M}$; non-competitive $IC_{50}^{NC,D}$ and $IC_{50}^{NC,M}$; uncompetitive $IC_{50}^{U,D}$ and $IC_{50}^{U,M}$; mixed type (1) $IC_{50}^{M_1,D}$ or mixed type (2) $IC_{50}^{M_2,D}$ and mixed type (1) $IC_{50}^{M_1,M}$, and mixed type (2) $IC_{50}^{M_2,M}$ with respect to monophenolase activity.

Step 4. Determination of i_D for $[D]_0 = 2k_m^D$ with a fixed concentration of inhibitor. The protocol is analogous to calculate the monophenolase activity, but in this case concentration of $[M]_0 = 2k_m^M$.

Step 5. Determination of the possible types of inhibition:

If the values of IC_{50}^{D} and $IC_{50}^{M}$are equal (IC_{50}^{D}=IC_{50}^{M})

The inhibition can be: competitive, non-competitive or uncompetitive $K_{I_3}^{app} = K_{I_2}^{app}$. Variation of substrate concentration:

A. $i_D^{2K_M^D} < i_D^{K_M^D}$ and $i_M^{2K_M^M} < i_M^{K_M^M}$, the inhibitor is competitive.
B. $i_D^{2K_M^D} = i_D^{K_M^D}$ and $i_M^{2K_M^M} = i_M^{K_M^M}$, the inhibitor is non-competitive.
C. $i_D^{2K_M^D} > i_D^{K_M^D}$ and $i_M^{2K_M^M} > i_M^{K_M^M}$, the inhibitor is uncompetitive with ($K_{I_3}^{app} = K_{I_2}^{app}$).

If the values of IC_{50}^{D} and IC_{50}^{M} are not equal ($IC_{50}^{D} \neq IC_{50}^{M}$)

The inhibition can be: uncompetitive ($K_{I_3}^{app} \neq K_{I_2}^{app}$), or mixed type (1) or mixed type (2),

D. If $i_D^{2K_M^D} < i_D^{K_M^D}$ and $i_M^{2K_M^M} < i_M^{K_M^M}$ the inhibition would be mixed type (1)
E. If $i_D^{2K_M^D} > i_D^{K_M^D}$ and $i_M^{2K_M^M} > i_M^{K_M^M}$ there is an ambiguity between uncompetitive and mixed inhibition type (2).

Step 6. Obtention of the kinetic information in the cases in which $IC_{50}^{D} = IC_{50}^{M}$:

Case A) Competitive inhibitor: the kinetic information ($K_{I_1}^{app}$) is obtained by applying Eq. (S16) for diphenolase activity and Eq. (S23) for monophenolase activity. Note that in both cases $K_{I_1}^{app}$ is obtained.

Case B) Non-competitive inhibition: the kinetic information $\left(K_I^{app}\right)$ is obtained by applying Eq. (S18) in the case of diphenolase activity and Eq. (S36) in the case of monophenolase activity. Both values must be the same.

Case C) Uncompetitive inhibition: the kinetic information $\left(K_{I_2}^{app}\right)$ is obtained by applying Eq. (S19) for diphenolase activity and Eq. (S26) for monophenolase activity. In this case $K_{I_3}^{app}$ is determined, which can be the same as $K_{I_2}^{app}$ or different.

Step 7. Distinction between the ambiguous uncompetitive inhibition and mixed (type 2).

i_D values at different concentrations of $[D]_0$ and fixed concentration of inhibitor should be obtained.

The same procedure is followed with the i_M determination at different concentrations of $[M]_0$ at a fixed concentration of inhibitor.

Representation of i_D and i_M with respect to the value n. If hyperbolas are obtained that pass through the origin of coordinates when representing i_D vs n_D, the inhibitor would be of the uncompetitive type and the kinetic information is obtained through the non-linear regression adjustment to Eq. (S39), obtaining the value of $K_{I_2}^{app}$. An analogous protocol is carried out in the case of monophenolase activity, accomplishing a non-linear regression of i_M with respect to n_M which originates hyperbolas that pass through the origin of coordinates and allows the determination of $K_{I_3}^{app}$. In the case of mixed type inhibition (2), the hyperbolas i_D vs n_D o i_M vs n_M never pass through the origin of coordinates.

Step 8. Obtention of the kinetic information in the case of mixed type (1) or type (2) inhibitors.

In the representation of i_D against n_D in case *D*, descending hyperbolas are obtained that do not pass through the origin of coordinates. Non-linear regression analysis of i_D against n_D according to Eq. (S47)

provides the values of $K_{I_1}^{app}$ and $K_{I_2}^{app}$. In the case of monophenolase activity, the analysis of the values of i_M with respect to n_M according to Eq. (S51) provides the values of $K_{I_1}^{app}$ and $K_{I_3}^{app}$.

In the case of mixed type (1) or mixed type (2) inhibitors, the values of the calculated inhibition constants must be checked, verifying compliance with Eqs. (S20) and (S27) for the values of IC_{50}^{D} and IC_{50}^{M}.

2.4. Considerations on the experimental data described in the bibliography

For the discussion of the different inhibitors, the data have been grouped into five Tables, dividing them according to the type of inhibition. Competitive (Table 1), non-competitive (Table 2), uncompetitive (Table 3) and mixed (Tables 4A and B). The inhibitors described in these tables have been characterized using mushroom tyrosinase, which is the commercial enzyme used as a model. In addition, Tables 5A and B describe the action of inhibitors in a comparative way on the enzymes of *S. antibioticus, A. bisporus and Homo sapiens*.

The data available in the bibliography are considered in accordance with the focus of this work, i.e. with regard the kinetic study leading to the calculation of the inhibition type and its strength. It should be noted that most of the data correspond to diphenolase activity. The data on monophenolase activity are described when they exist. In any case, the reported experimental data on monophenolase activity described herein have not been carried out with the experimental design proposed by our research group [15,16,18]. As previously described, a concentration of L-dopa is necessary to reach steady state at $t = 0$, fulfilling the relationship $[D]_{ss}/[M]_{ss} = R$ [14]. Considering the above-mentioned, the monophenolase activity data may not be rigorous since in the presence of an inhibitor there is no assurance that the system has reached steady state with a given rate.

The inhibitors listed in each table are described below.

2.4.1. Competitive inhibitors

The mushroom tyrosinase competitive inhibitors described in this critical review article are shown in Table 1.

Competitive inhibitors of tyrosinase are the most numerous and due to the broad substrate specificity of the enzyme, they are the simplest to design. The determination of the IC_{50}^{M} and IC_{50}^{D} parameters can help its kinetic characterization. The kinetic analysis described in the Supplementary Material predicts that in this case (competitive inhibitors) $IC_{50}^{M} = IC_{50}^{D}$. To ratify that the inhibition is of a competitive type, an experiment can be carried out with double the concentration of substrate, L-dopa, that should produce a decrease in the value of i_D. The inhibition constant $K_{I_1}^{app}$ can be calculated from Eqs. (S16) for diphenolase activity or (S23) for monophenolase activity since this constant is common to both activities.

When the data in Table 1 are analyzed, it can be concluded that most

Table 1
Tyrosinase inhibitors described as competitive. Monophenolase and diphenolase activities. Values of the IC_{50} parameter and its relationship with the inhibition constant. $IC_{50} = (1 + n)\ K_I^{app}$. With $n_M = \frac{[M]_0}{K_m^M}$ and $n_D = \frac{[D]_0}{K_M^D}$.

Compound name	Monophenolase activity			Diphenolase activity			References
	$IC_{50}^{C,M}(\mu M)$	n_M	$K_{I_1}^{app}(\mu M)$	$IC_{50}^{C,D}(\mu M)$	n_D	$K_{I_1}^{app}(\mu M)$	
1) Cupferron	0.52	2	0.20	0.84	0.75	0.48	[4]
2) Quercetin	–	–	–	50	1	29	[5]
3) Galangin	–	–	–	101	1	58	"
4) Fisetin	–	–	–	130	1	75	"
5) 3,7,4′-Trihydroxyflavone	–	–	–	240	1	154	"
6) Morin	–	–	–	720	1	410	"
7) Ferulic acid	–	–	–	150	1	250	[6]
8) 4-carboxylphenol	–	–	–	1030	1	750	"
9) 4-formylphenol	–	–	–	1150	1	690	"
10) 4-cyanophenol	–	–	–	800	1	310	"
11) 4-hexylresorcinol	–	–	–	850	1	443	"
12) 4,4′-dihydroxybiphenyl	–	–	–	1910	1	400	"
13) Arbutin	–	–	–	5300	1	2980	"
14) 2-fluorobenzaldehyde	1350	1	920	1620	1	920	[7]
15) Cuminaldehyde	120	1.4	50	n.c	n.c	n.c	[8]
16) Kazinol C	15.5	1	10.8	22.8	1	11.2	[9]
17) Kazinol F	0.96	1	0.41	1.7	1	0.77	"
18) Broussonin C	0.43	1	0.23	0.57	1	0.29	"
19) Kazinol S	17.9	1	9.12	26.9	1	15.6	"
20) Isooctyl 4-hydroxy-3-methoxycinnamate	240	2	450	450	1	200	[10]
21) Isoferulic acid	130	2.5	110	390	2.5	110	[11]
22) 3-Hydroxy-4-methoxycinnamic Acid	–	–	–	320	1.56	120	[12]
23) Ferulic acid isooctyl	–	–	–	450	1.1	200	"
24) Methyl cinnamate	–	–	–	1490	1.36	660	"
25) Cinnamic alcohol	–	–	–	2080	1	760	[12]
26) Quercetin	–	–	–	130	2.38	38	"
27) 4-hexylresorcinol	–	–	–	0.87	1	0.44	"
28) 4-dodecyleresorcinol	–	–	–	0.82	1	0.40	"
29) Safrole	32.11	1	16.05	27.32	1	13.66	[13]
30) Cajanin	–	–	–	29.9	1	55.9	[14]
31) 2-((Benzo[*d*]thiazol-2-ylthio)methyl)-5-hydroxy-4H-pyran-4-one	–	–	–	3.23	1	1.96	[15]
32) (*E*)-4′-metoxy acetophenone thiose micarbazone	–	–	–	1.8	1	0.6	[16]
33) (*E*)-4′-hydroxy acetophenone thiose micarbazone	–	–	–	0.80	1	0.6	"
34) (*E*)-3′-nitro acetophenone thiose micarbazone	–	–	–	14.2	1	0.4	"
(35) ((Z)-2-(benzylamino)-5-(3-hydroxy-4-methoxybenzylidene)thiazol-4(5H)-one	10.0	2	3.1	10.3	2	3.1	[17]
(36) ((*Z*)-2-(benzylamino)-5-(2,4-dihydroxybenzylidene)thiazol-4(5H)-one	0.27	2	0.81	1.04	2	0.81	"
(37) 3-fluoro-hydroxypyranone-thiosemicarbazone	–	–	–	1.96	2	3.42	[18]
(38) Benzoic Acid	1.06	1	0.53	0.98	1	0.49	[19]
(39) Cinnamic Acid	0.92	1	0.46	0.80	1	0.40	[19]

n.c. = non-competitive.

Table 2
Tyrosinase inhibitors described as non-competitive. Monophenolase and diphenolase activities. Values of the IC_{50} parameter and its relationship with the inhibition constant. $IC_{50} = K_I$.

Compound name	Monophenolase activity			Diphenolase activity			References
	$IC_{50}^{NC,M}(\mu M)$	n_M	$K_{I_1}^{app}(\mu M)$	$IC_{50}^{NC,D}(\mu M)$	n_D	$K_{I_1}^{app}(\mu M)$	
1) 4-fluorobenzoic acid	1030	–	–	260	–	250	[20]
2) 4-chlorobenzoic acid	750	–	–	200	–	200	"
3) 4-bromobenzoic acid	500	–	–	180	–	170	"
4) Cuminaldehyde	50	–	120	50	–	9	[8]
5) Cinnamic acid	–	–	–	610	–	580	[12]
6) Cinnamonitrile	–	–	–	620	–	640	"
7) Benzaldehyde	–	–	–	800	–	810	"
8) Biphenyl formaldehyde	–	–	–	90	–	90	"
9) *m*-Methoxybenzaldehyde	–	–	–	1600	–	1780	"
10) *p*-Methoxybenzaldehyde	–	–	–	350	–	350	"
11) 2-O-β-glucopyranosyloxy-4-methoxy-hydrocinnamic acid	224.1	–	72.79	–	–	–	[21]
12) Dihydromelilotoside	311.9	–	169.32	–	–	–	"
13) 2-O-β-glucosyloxy-4-methoxy trans-cinnamic acid	369.9	–	109.89	–	–	–	"
14) (6aR,11aR)-3-Hydroxy-9,10-dimethoxypterocarpan	24.6	–	24.9	–	–	–	[14]
15) (6aR,11aR)-3,8-Dihydroxy-9,10-dimethoxypterocarpan	59.2	–	42.2	–	–	–	"
16) 3-(1-(4-Hydroxy-3-methoxyphenyl)propyl)-benzene-1,2-diol	–	–	–	2.32	–	2.95	[22]
17) 4-(1-(2-Hydroxy-4-methoxyphenyl-)propyl)-2-methoxyphenol	–	–	–	2.83	–	3.74	"

Table 3
Tyrosinase inhibitors described as uncompetitive. Monophenolase and diphenolase activities. Values of the IC_{50} parameter and its relationship with the inhibition constant. $IC_{50} = (1+\frac{1}{n})\,K_{I_2}^{app}$. With $n_M = \frac{[M]_0}{K_M^M}$ and $n_D = \frac{[D]_0}{K_M^D}$.

Compound name	Monofenolase activity			Difenolase activity			References
	$IC_{50}^{U,M}(\mu M)$	n_M	$K_{I_1}^{app}(\mu M)$	$IC_{50}^{U,D}(\mu M)$	n_D	$K_{I_1}^{app}(\mu M)$	
1) *p*-tolualdehyde	–	–	–	120	0.47	51.5	[23]
2) *p*-ethylbenzaldehyde	–	–	–	95	0.47	40.8	"
3) *p*-propylbenzaldehyde	–	–	–	75	0.47	32.2	"
4) *p*-isopropylbenzaldehyde	–	–	–	75	0.47	32.2	"
5) *p*-tert-butylbenzaldehyde	–	–	–	38	0.47	16.3	"
6) *p*-butyl-benzaldehyde	–	–	–	38	0.47	16.3	"
7) *p*-pentylbenzaldehyde	–	–	–	13.5	0.47	5.8	"
8) *p*-hexylbenzaldehyde	–	–	–	8	0.47	3.4	"
9) *p*-heptylbenzaldehyde	–	–	–	8.8	0.47	3.8	"
10) *p*-octylbenzaldehyde	–	–	–	10	0.47	4.3	"
11) Luteolin	–	–	–	240	0.47	103	[5]
12) (3*R*)-7-Hydroxy-3′,4′-dimethoxy isoflavane	42.4	1	22.1	–	–	–	[14]

of the data correspond to diphenolase activity.

For compound (1) [28], the inhibition studies show good relationships of IC_{50}^{M} and IC_{50}^{D} in relation to the values of $K_{I_1}^{app}$. However, the values of $K_{I_1}^{app}$ in the two activities are slightly different. The simplest explanation could correspond to the fact that the monophenolase activity data have not been carried out by adding L-dopa to the medium, so that the steady state is not reached at $t = 0$ [15,16,18].

The data describing the action of inhibitors described in Table 1, compounds (2–13) [29,30] practically show an acceptable relationship between $IC_{50}^{C,D}$ and K_I^{app} except for compound (7) [30], ferulic acid, which is behaves like an alternative substrate and shows a deviation from the quantitative behavior that is reflected in the $IC_{50}^{C,D} < K_I^{app}$.

The compounds (14,16–19) [31,32] show kinetic behavior in accordance with Eqs. (S13) and (S23). However, compound (15) [33] gives an anomalous behavior, since it cannot be competitive with respect to monophenolase activity and non-competitive with respect to diphenolase activity [33].

The compounds (20,21) [34,35] show anomalous kinetic behavior especially with respect to monophenolase activity; these compounds could be alternative substrates.

With respect to compounds (22–28) [36], the kinetic behavior regarding diphenolase activity practically conforms to Eq. (S16).

Compounds (29 and 35) [37–41] give rise to $IC_{50}^{C,D}$ and $IC_{50}^{C,M}$ kinetic data that fit Eqs. (S13) and (S23). However, compound (36) [41] does not fit the Eqs., especially in the monophenolase activity, which shows an $IC_{50}^{C,M} < K_I^{app}$.

Regarding the inhibition of diphenolase activity, compounds (31–34) [39,40] approximately comply with Eq. (S13), however, compound (37) [42] does not fit, since $IC_{50}^{C,D} < K_I^{app}$.

The compounds (38 and 39) [16] show values of $IC_{50}^{C,D}$ and $IC_{50}^{C,M}$ that quantitatively correlate with Eqs. (S13) and (S23). It should be noted that the monophenolase activity data have been carried out by adding to the reaction mixture an amount of *o*-diphenol that meets the relationship $[D]_{ss} = R[M]_{ss}$ [14] and in this case, $R = 0.1$ [16], according to the experimental design proposed in this review article.

In general, different possible causes of deviations can be considered:

a) Deviations are more frequent in measurements of monophenolase activity.
b) The $K_{I_1}^{app}$ values determined from the monophenolase activity measurements are lower than those determined from the diphenolase activity. The presence of the delay period and the measurement, at all inhibitor concentrations, on the same time scale, makes the velocity values determined for this activity lower than the real ones, because the system has not reached steady state.
c) Another source of error could be that the compounds studied as inhibitors are alternative substrates of the enzyme [17], as is the case of the compound (7) ferulic acid [30].

Table 4A
Tyrosinase inhibitors described as mixed type 1 or type 2. Monophenolase and diphenolase activities. Values of the IC50 parameter and its relationship with the inhibition constant $K_{I_1}^{app}$ and $K_{I_2}^{app}$ and $K_{I_3}^{app}$. $IC_{50}^{M} = K_{I_1}^{app} K_{I_3}^{app} (1 + n_M)/(K_{I_3}^{app} + n_M K_{I_1}^{app})$; $IC_{50}^{D} = K_{I_1}^{app} K_{I_2}^{app} (1 + n_D) / (K_{I_2}^{app} + n_D K_{I_1}^{app})$

Compound name	Monofenolase activity	Difenolase activity				References
	$IC_{50}^{M,M}(\mu M)$	$IC_{50}^{M,D}$(μM)	n_D	$K_{I_1}^{app}$ and $K_{I_2}^{app}(\mu M)$		
1) *o*-Tolualdehyde	–	2600	1.25	1853	240	[23]
2) *m*-Tolualdehyde	–	450	1.25	4775	880	"
3) 4-Cyanobenzaldehyde	620	720	0.9	507	1354	[24]
4) 4-Cyanobenzoic acid	2450	1400	1.5	1543	1321	"
5) 4-Vinylbenzaldehyde	93	23	0.5	29.7	16.4	[25]
6) 4-Vinylbenzoic acid	3010	330	1.3	540	220	"
7) 3-Fluorobenzaldehyde	1800	1060	0.75	680	3990	[7]
8) 4-Fluorobenzaldehyde	1050	160	0.7	110	450	"
9) 4-Hydroxy-3-methoxybenzoic acid	1300	2600	0.68	1760	8570	[26]
10) *p*-Coumaric acid	–	500	3.3	290	600	[12]
11) Cinnamaldehyde	–	1050	0.7	770	1560	"
12) 4-Hydroxy-3-methoxybenzoic acid	–	2750	1.6	1350	7170	"
13) 2′-Hydroxy-4′-methoxyacetophenone	–	600		80	120	[27]
14) 1-Hexyl-5-hydroxy-4-oxo-1,4-dihydropyridine-2-carbaldehyde O-ethyl oxime	2.04	1369		24.84	32.54	[28]
15) 5-Hydroxy-1-octyl-4-oxo-1,4-dihydropyridine-2-carbaldehyde O-ethyl oxime	1.60	7.99	–	18.07	21.34	"
16) (*E*)-1-(2-hydroxy-5-methylbenzylidene)thiosemicarbazide	–	1.02	–	1.81	32.38	[29]
17) (*E*)-1-(3-((benzylethylamino)methyl)-2-hydroxy-5-methyilbenzylidene)thiosemicarbazide	–	143.56	–	13.06	17.28	"
18) (*E*)-1-(3-((diethyilamino)methyl)-2-hydroxy-5-methylbenzylidine)thiosemicarbazide	–	87.45	0.73	12.31	18.95	"
19) (*E*)-1-(3-((ethyl-2-hydroxyethylamino)methyl)-2-hydroxy-5-methyl-benzylidene) thiosemicarbazide	–	10.45	–	6.16	8.72	"
20) (*E*)-1-(3-(bis(2-hydroxyethylamino)methyl)-2-hydroxy-5-methyl-benzylidene) thiosemicabazide	–	27.41	0.5	5.15	9.08	"
21) (*E*)-1-(3-((benzyl-2-hydroxyethylamino)methyl)-2-hydroxy-5-methyl-benzylidene) thiosemicarbazide	–	4.85	0.16	4.37	16.23	"
22) (*E*)-3′-Methoxyacetophenone thiosemicarbazone	–	1.9	1.1	1	9.7	[16]
23) (*E*)-3′-Aminoacetophenone-micarbanzone	–	0.65	0.1	0.6	3.05	"
24) (*E*)-3′-Hydroxyacetophenone thiosemicarbazone	–	0.95	0.7	0.6	5.4	"
25) (*E*)-2′-Nitroacetophenone thiosemicarbazone	–	154.5	–	8.6	43.3	"
26) (*E*)-4′-nitroacetopheone thiosemicarbazone	–	1.6	–	5.0	27.5	"
27) 2-(3,5-dihydroxystyryl-hexyl-5-hydroxy piridin-4(16) one	2.72	15.86	–	40.90	50.25	[30]
28) 2-((C5,6-Bis (2-Bromophenyl)-1,2,4-triazin-3-yl)thio)methyl)-5—hydroxy-4H pyran-4-one	–	0.34	–	0.73	3.27	[31]

Table 4B
Tyrosinase inhibitors described as mixed type. Parameters IC_{50}^{M} and IC_{50}^{D}. Values of the inhibition constants in monophenolase and diphenolase activity.

Compound Name	Monofenolase activity				Difenolase activity				References
	$IC_{50}^{U,M}$	n_M	$K_{I_1}^{app}$	$K_{I_3}^{app}$	$IC_{50}^{U,D}$	n_D	$K_{I_1}^{app}$	$K_{I_2}^{app}$	
1) Benzylideneacetone	1.5 mM (M)	1	0.89 mM	3.97 mM	2.0 mM (M)	0.5	1.73 mM	3.89 mM	[32]
2) Benzylacetone	2.8 Mm (M)	–	1.25 mM	0.52 mM	0.6 Mm (M)	0.47/1	0.39	1.33	"
3) 4-Phenyl-2-butanol	1.1 mM (M)	2.75	0.49 mM	1.83 mM	0.8 mM (N.C.)	0.47/5	1.02 mM		"
4) γ-Picolyl propyl amine	Uncompetitive				5.8 mM (M)	–	23 mM	37 mM	[33]
5) Nicotinic acid hydroxamate	0.2 (M)	–	1.04 mM	3.65	0.1 (uncompetitive)	–	1.23	–	[34]
6) Glabridin	80 nM (M)	–	13.6 nM	281 nM	294 nM (M)	0.47	57 nM	1312 nM	[35]
7) MEBA	31.96 μg/mL(M)	–	8.21 μg/mL	45.33 μg/mL	–	–	–	–	[36]
8) Puerarin	0.537 mg/mL(M)	3.7	0.15 mg/mL	1.76 mg/mL	Activation				[37]
9) Arbutin	–	–	–	–	Activation				[38]
	–	–	–	–	Alternative substrate				[39]

M = mixed; U = uncompetitive; N. C. = non –competitive; (7) = methanol extract of *Berberis aristata*.

2.4.2. Non-competitive inhibitors

Non-competitive inhibitors of mushroom tyrosinase described in this work are shown in Table 2.

This inhibition does not depend on the substrate concentration and the values of IC_{50}^{M} and IC_{50}^{D} must be equal. The non-competitive character implies that all the inhibition constants are equal to IC_{50}^{M} and IC_{50}^{D}, according to the kinetic analysis described in the Supplementary Material.

Compounds (1–3) [43] regarding diphenolase activity shown a kinetic behavior according to Eqs. (S18) and (S25). However, with respect to monophenolase activity, the behavior is not as expected from the kinetic studies, $IC_{50}^{M} > IC_{50}^{D}$. We attribute this issue to an inaccurate measurement of monophenolase activity.

Regarding compound (4) [33], although the values of IC_{50}^{M} are the same as IC_{50}^{D}, the values of $K_{I_1}^{app}$ are different. This observation is not in accordance with the kinetic studies (see Supplementary Material). The studies of diphenolase activity of compounds (5–10) [36] comply with Eq. (S18). In the case of compounds (11−13) [44], the data obtained do not comply with the kinetic equations, since $IC_{50}^{M} \neq K_{I_1}^{app}$. On the other hand, there is a correspondence in the case of monophenolase activity between IC_{50}^{M} and K_I^{app} in compounds (14,15) [38]. In the case of diphenolase activity for compounds (16,17) [45] there is a reasonably acceptable relationship between IC_{50}^{D} and K_I^{app}.

2.4.3. Uncompetitive inhibitors

Some uncompetitive inhibitors of mushroom tyrosinase described in the bibliography are shown in Table 3. This group is the minority in relation to the other types of inhibition. The study of compounds (1−11) [29,46] for diphenolase activity, comply with a good approximation of the kinetic Eqs. (S19) and (S26) described in the Supplementary Material. The effect of compound (12) [38] on monophenolase activity has been described. This data is collected in Table 3 and fulfils the corresponding kinetic equations.

2.4.4. Mixed inhibitors

A series of mixed-type inhibitors on mushroom tyrosinase are listed in Tables 4A and B.

It should be noted that most of the inhibitors correspond to diphenolase activity, Table 4A shows a series of mixed inhibitors, but the detailed study is only carried out with diphenolase activity, with monophenolase activity only the data shown in Table 4B. Table 4A offers the values of $IC_{50}^{M_1,D}$(mixed, diphenolase) which are related to the values of $K_{I_1}^{app}$ and $K_{I_2}^{app}$, and also with the parameter n_D that corresponds to the relationship of the concentration of substrate to the Michaelis constant $n_D = \frac{[D]_0}{K_M^D}$.

The inhibitors described in these Tables are:

Compounds (1,2) [46], compounds (3,4) [47], compounds (5,6) [48], compounds (7,8) [31], compound (9) [49], compounds (10−12) [36], compound (13) [50], compounds (14–15) [51], compounds (16,21) [52], compounds (22,26) [40], compound (27) [53], compound (28) [54].

From these data it can be concluded that:

a) Most of the data described indicate that $IC_{50}^{M_1,D} > IC_{50}^{M_1,M}$(mixed, monophenolase), so they are different $IC_{50}^{M_1,M} \neq IC_{50}^{M_1,D}$.
b) Most of the mixed inhibitors are type 1 with $K_{I_2}^{app} > K_{I_1}^{app}$.
c) Regarding the calculation of the value n_D, from the $IC_{50}^{M_1,D}$ and the inhibition constants, of the examples included in Table 4A, a value of $n < 0$ is obtained in many of the cases. This calculation could indicate that the values of $IC_{50}^{M_1,D}$, $K_{I_1}^{app}$ and $K_{I_2}^{app}$ are not completely accurate.
d) Another group of mixed type inhibitors is shown in Table 4B. In this group, data on the inhibition of monophenolase activity are shown in more detail and also data on the inhibition of diphenolase activity is presented. According to the kinetics of the inhibition on both activities the following information can be obtained:

Monophenolase activity: $IC_{50}^{M_1,M}$, $K_{I_1}^{app}$ and $K_{I_3}^{app}$.

Diphenolase activity: $IC_{50}^{M_1,D}$, $K_{I_1}^{app}$ and $K_{I_2}^{app}$.

The inhibitors considered are described in Table 4B, being the following:

Compounds (1–3) [55], compound (4) [56], compound (5) [57], compound (6) [58], compound (7) [59], compound (8) [60] and compounds (9) [61,62]

As described below, among the data shown, only one case is described in which the data fit the equations.

The problem with the monophenolase activity of the enzyme is the presence of a lag period that makes steady state rate determinations difficult. To avoid the ambiguity, we propose to add a concentration of L-dopa that is necessary for the system to reach the steady state at $t=0$ [15,16].

2.4.5. Action of different inhibitors on tyrosinases from different sources

Tables 5A and B show the kinetic results of the action of different inhibitors on enzymes from different sources (*Streptomyces antibioticus*, (ScTyr), *Agaricus bispurus,* (mTyr) *and Human Tyrosinase* (hTyr)) [63].

The data described in Table 5A show that the inhibitors have specific properties depending on the source of the enzyme. Thus, compound (1) [63] inhibits hTyr with a 100-fold greater potency than mTyr. However, compounds (2–8) [63] inhibit mTyr with more potency. The comparison of the IC_{50}^{D} values of hTyr and mTyr indicates with which enzyme a certain inhibitor acts better when the experiments are done at the same ratio ($[D]_0/K_M^D = n$). It should be noted that in the case of hTyr the IC_{50}^{D} values are related to K_I^{app} by Eq. (S-16). In the case of compound (1) [63], the application of this equation to the values of IC_{50}^{D} and K_I^{app} provides the values of the experimental parameter n_D indicated below: compound (1) [63] $n = 3$, compound (2) [63] $n = 1.3$, compound (3) [63] $n = 1.4$ and compound (4) [63] $n = 4.4$. These values could indicate that compounds (1 and 4) [63] behave as alternative substrates, because they would act as inhibitors and as substrates, but catalysis can be followed experimentally only in the action on L-dopa, with which the value of IC_{50}^{D} is higher or that these compounds (1 and 4) [63] have been studied at a high ratio of $[D]_0/K_M^D$.

In the data presented in Table 5B regarding a series of compound inhibitors (1–6) [64] and three types of enzymes which are compared, the lowest values of K_I^{app}, and therefore the strength of the inhibitor, correspond to TyAb and TySa. This set of inhibitors behaves with different values of K_I^{app} and even the type of inhibition changes, as occurs with compounds (2, 5 and 6) [64].

Table 5A
Action of different inhibitors on tyrosinases from different sources (*Agaricus bisporus*,mTyr and *Human Tyrosinase*, hTyr). diphenolase activity. Competitive.

Compound	$IC_{50}^{U,D}$ (μM)		$K_{I_1}^{app}$ (μM)	References
	hTyr	mTyr	hTyr	
1) Thiamidol	1.1	108	0.25	[40]
2) 4-Butylresorcinol	21	0.6	9.1	"
3) 4-Hexylresorcinol	94	1.2	39	"
4) 4-Phenylethylresorcinol	131	0.3	24	"
5) Kojic acid	500	6.0	145	"
6) Hydroquine	>4000	1.1	n.d.	"
7) Arbutin	>4000	40	n.d.	"
8) Dimethoxytolylpropyl resorcinol	No inh	0.24	n.d.	"

(n.d. not determined; no inh, not inhibition).

3. Conclusions

The study of inhibitors of the tyrosinase enzyme is a research topic of

Table 5B
Action of different inhibitors on tyrosinases from different sources (*Streptomyces antibioticus*, TySa, *Agaricus bisporus*, TyAb and *Homo sapiens Tyrosinase*, TyHs) Diphenolase activity.

Compound	$IC_{50}^{U,D}$ (μM)		$K_{I_1}^{app}$ (μM)			References
	TySa	TyAb	TyAb	TySa	TyHs	[41]
1) HOPNO	6.4 ± 1.1	1.2	1.8 (C)	7.7 ± 0.2 (C)	128 ± 2 (C)	"
2) PTSC	24 ± 2	7.5 ± 0.5	0.93 ± 0.1(C)	$K_{ic\ =}$ 19 ± 2 (M) $K_{iu=}$ 30 ± 9	n.a.	"
3) KA-TSC	1.6 ± 0.2	9.6 ± 0.8	7.2 ± 0.4 (C)	1.4 ± 0.4 (C)	260 ± 20 (nC)	"
4) HOPNO-TSC	2.0 ± 0.5	0.56 ± 0.03	0.5 ± 0.1 (C)	0.82 ± 0.9 (C)	47 ± 1 (C)	"
5) 4PyNO-TSC	10 ± 2	18 ± 2	$K_{ic\ =}$ 58 ± 14 (M) $K_{iu=}$ 98 ± 22	7 ± 2 (C)	1800 ± 200 (nC)	"
6) 3PyNO-TSC	24 ± 5	60 ± 3	$K_{ic\ =}$ 69 ± 9 (M) $K_{iu=}$ 108 ± 34	13 ± 3 (C)	3100 ± 200 (nC)	"

(n.a, Not applicable; C, competitive; M, mixed, nC, non-competitive).

great interest due to the involvement of this enzyme in highly relevant processes, such as pigmentation and the browning of fruits and vegetables, as well as its applications in cosmetics. An experimental design is proposed that allows the characterization of the type and strength of the inhibitor under study. The study of the two activities of the enzyme, monophenolase and diphenolase, allows to characterize the degrees of inhibition i_M and i_D. Their dependence on the concentration of inhibitor allows to determine the parameters IC_{50}^{M} and IC_{50}^{D}. Using these values and establishing a comparison, the type (competitive, non-competitive, uncompetitive or mixed) and strength of a tyrosinase inhibitor can be determined (K_I). Additionally, an analysis of different types of inhibition described in the literature, mainly for the mushroom enzyme, has been carried out, considering our proposed analysis and information on tyrosinase from other sources. These data lead to deduce that, in order to obtain more accurate kinetic parameters of monophenolase activity, a necessary amount of *o*-diphenol should be added at the beginning of the reaction to reach the steady state in a very short time. If this experimental design is not carried out, it is not known whether the system reaches steady state and, therefore, the initial velocity values may be imprecise. This review article aims to establish a suitable experimental design that helps researchers in the field to accurately determine both the monophenolase and diphenolase activities.

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Declaration of competing interest

The authors declare no competing financial interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.131513.

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